

Production of hemopexin by TNF- α stimulated human mesangial cells

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Background. Plasma hemopexin has been shown to induce proteinuria after intrarenal infusion in rats, as well as glomerular alterations identical to those seen in corticosteroid-responsive nephrotic syndrome (CRNS). The question emerged whether also renal cells are potentially able to release hemopexin.

Methods. Normal human mesangial cells (HMC) were incubated overnight in serum-free medium with or without tumor necrosis factor- α (TNF- α) (10 ng/mL). Parallel cultures were supplemented with prednisolone (10^{-3} mol/L). Concentrated supernatants were analyzed by Western blotting, using anti-hemopexin immunoglobulin G (IgG). Antitransferrin IgG served as control antibody. In addition, cytopins were stained using polyclonal or monoclonal antihemopexin IgG. A part of the cells was used for RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR), to study hemopexin mRNA.

Results. Eighty five kD bands were exclusively detected by antihemopexin IgG in the Western blots in supernatants from TNF- α -stimulated cultures and to a lesser extent in prednisolone-treated cultures. Cells from TNF- α -stimulated cultures stain positive for hemopexin in contrast to those from prednisolone-treated or nonstimulated cultures. RT-PCR data suggest that mRNA for hemopexin is up-regulated in TNF- α -treated versus prednisolone-treated HMC.

Conclusion. Stimulated HMC are able to release hemopexin in vitro in a corticosteroid-dependent manner. As preliminary data indicate that mesangial hemopexin is able to affect glomerular anionic sites, it is conceivable that stimulated mesangium may contribute to enhanced glomerular permeability in CRNS through local hemopexin release.

Hemopexin is an acute phase reactant present in the mammalian and human circulation [1, 2]. Hemopexin, which is known as a scavenger of heme, is like other acute

phase reactants produced by hepatocytes [3, 4]. However, recently, other sites of hemopexin production have been detected (i.e., mononuclear blood cells [5], retina cells, and in neural tissue [6–10]). It has also been recently shown from studies with hemopexin-deficient mice that the most significant role of hemopexin is that of protecting cells against heme toxicity rather than participating in iron metabolism [11].

Previously, we proposed that hemopexin may act as a potential proteinuria-promoting factor associated with corticosteroid-responsive nephrotic syndrome (CRNS) [12]. This association was based upon the observation that CRNS-like glomerular lesions as well as proteinuria could be induced after intrarenal infusion of hemopexin into the rat [13]. Identical effects are seen following infusion of recombinant human hemopexin into rats (to be reported). In addition, following contact with renal tissue in vitro, this active isoform of hemopexin is able to induce identical glomerular alterations (i.e., loss of anionic sites and loss of glomerular ecto apyrase expression), whereas this activity could be inhibited by serine protease inhibitors but not by collagenase inhibitors [12]. In patients with CRNS in relapse versus remission, the plasma hemopexin level is decreased, whereas urinary excretion of this protein could be excluded, suggesting that increased turnover of hemopexin may have occurred in these subjects [14] (abstract; Cheung PK, *J Am Soc Nephrol* 9:85A, 1998).

As it has been shown in experimental animals that hemopexin can be produced locally by various cells outside the liver after proinflammatory stimulation [5], the question arises whether besides circulating hemopexin, intraglomerular-generated hemopexin might also be produced by human mesangial cells.

In the present communication, we show that human mesangial cells in vitro are able to release hemopexin, in particular after stimulation with inflammatory cytokines like tumor necrosis factor- α (TNF- α). In addition, it ap-

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pears that mesangial hemopexin production *in vitro* could be inhibited by treatment of the cells with prednisolone.

METHODS

Experimental design

Confluent mesangial cell cultures were washed with RPMI 1640 medium and incubated in serum-free medium overnight with or without TNF- α (10 ng/mL). Parallel cultures were supplemented with prednisolone (10^{-3} mol/L). Culture supernatants were subsequently harvested, concentrated, and examined for the presence of hemopexin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using rabbit antihemopexin immunoglobulin G (IgG). Antihuman transferrin IgG served as control antibody to check for nonspecific staining.

Cells were carefully detached from the culture flasks using a cell scraper, washed, and cytopins were prepared, fixed with acetone and stained for hemopexin using monoclonal or polyclonal antihemopexin IgG, or antitransferrin as a control antibody.

A part of the cells of the primary cell cultures was used for mRNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) to study the mRNA signal for hemopexin.

Culture of human mesangial cells

Human mesangial cells were obtained from normal kidney cortices of patients undergoing unilateral nephrectomy for renal cell carcinoma. Only parts of the cortex with normal glomerular morphology, as evaluated by routine staining with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) and histochemical staining for sialoglycoproteins using colloidal iron [15], were used. Isolation of mesangial cells was done as described previously [16]. Briefly, glomeruli from the cortex of human kidney tissue were isolated by a gradual sieving procedure. Glomeruli were plated out onto gelatin-coated wells and then attached themselves within a few days. Outgrowth of predominantly epithelial cells and a limited number of endothelial cells were noted. Within 10 to 30 days after the glomeruli were seeded, outgrowth of mesangial was observed. Mesangial cells were selectively collected by scraping them from the tissue culture plate using a disposable cell scraper and subsequently expanded on gelatin-coated wells. To purify the mesangial cell populations further, an immunomagnetic separation technique was used. The cultured mesangial cells showed no immunoreactivity to antiplatelet-endothelial cell adhesion molecule-1 (PECAM-1) antibody or the anticytokeratin-8 antibody, excluding the presence of endothelial or epithelial cells. Cultured human mesangial cells were studied between passages 5 and 10.

Cells were cultured in RPMI 1640 medium (Gibco

BRL, Eggenstein, Germany) supplemented with 10% heat inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mmol/L glutamine. Subcultures were performed using trypsin/ethylenediaminetetraacetic acid (EDTA) and cells were plated in 75 cm² flasks (Costar, Corning, NY, USA). Confluent cultures were washed with culture medium and incubated overnight in serum-free medium supplemented with either TNF- α (10.0 ng/mL), a combination of TNF- α (10.0 ng/mL) and prednisolone (10^{-3} mol/L) or prednisolone alone (10^{-3} mol/L).

Subsequently cultures were carefully removed from the bottom of the culture flasks, counted (microcell-counter F800 Sysmex) and cytopins were prepared according to standard procedures. Cell viability, as measured by dye exclusion, was approximately 97%. A part of the cells were frozen at -80°C and used for RNA isolation. Supernatants were harvested and concentrated using Amicon B15 blocks (Millipore Corporation, Bedford, MA, USA).

Immunostaining of mesangial cells

Cytopsin preparations were fixed with ice-cold acetone (100%; -20°C) for 10 minutes, air dried and subsequently stained for hemopexin, using either rabbit antihemopexin IgG (Dako A/S, Glostrup, Denmark) and goat antirabbit IgG conjugated with peroxidase (Garpo; Dako) as a second step, or monoclonal antihemopexin IgG, and peroxidase-conjugated rabbit antimouse IgG (Rampo; Dako), as a second step, followed by Garpo as a third step. (Antihemopexin monoclonal antibody was a gift from Dr E. Hansen, the University of Texas Southwestern Medical Center at Dallas). The reaction product was visualized using 3-amino-9-ethyl-carbazole according to standard methods [17]. Cells stained for transferrin using rabbit antihuman transferrin IgG (Behring, Marburg, Germany) served as a control antibody. The sections were counterstained using hematoxylin.

Analysis of culture supernatants

Culture supernatants were dialysed against PBS pH 8.0 and run over gradient PAGE (4% to 15%; BioRad Laboratories, Hercules, CA, USA) and the proteins were visualized with Ponceau-S (Pharmacia, Uppsala, Sweden), together with prestained marker molecules (BioRad Laboratories) according to standard methods. Western blots were prepared and stained with either rabbit antihemopexin IgG, or control antibodies (antitransferrin IgG), Garpo, and peroxidase-conjugated rabbit antigoat IgG (Ragpo; Dako) as a second and third step, respectively. To evaluate the specificity of antihemopexin monoclonal antibody, this antibody was used to detect purified plasma hemopexin in the Western blot. Matrix metalloproteinase-2 (MMP-2) and MMP-9 (Neomarkers, Fremont, CA, USA; Sigma, Zwijndrecht, The

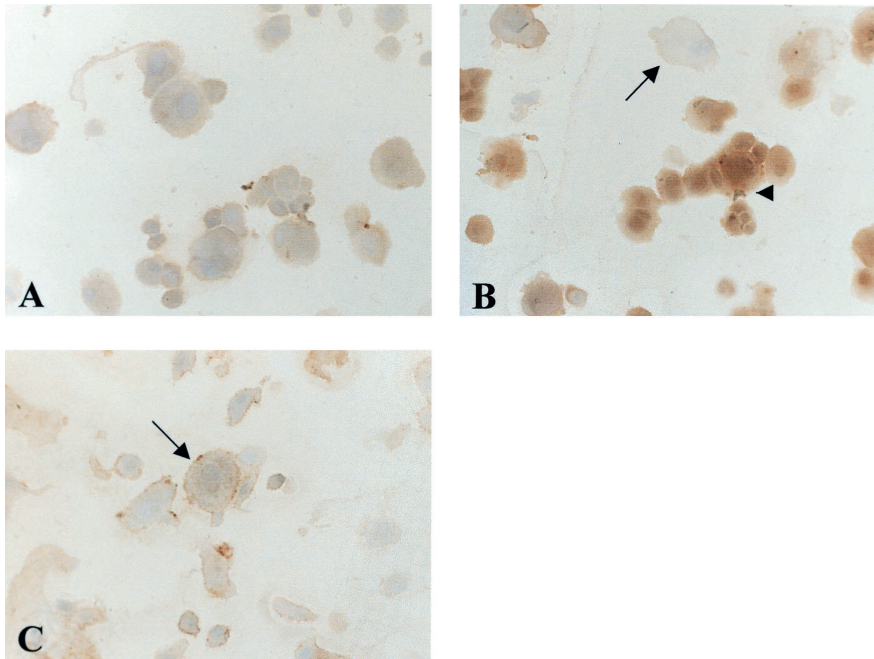


Fig. 1. Photomicrographs of mesangial cells following culture in serum-free conditions without stimulation (A), after stimulation by tumor necrosis factor- α (TNF- α) (B) or with TNF- α and prednisolone (C). Cytospins preparations were stained for hemopexin using rabbit anti-hemopexin IgG and peroxidase-conjugated goat antirabbit as a second step. Reaction product throughout the cytoplasm is seen in most stimulated cells (B, arrowhead); negative cells are also present (arrow). Nonstimulated cells stain predominantly negative (A), whereas stimulated cells after prednisolone treatment show also reduced stainability, although some membrane staining can be observed in these cells (C, arrow). Final magnification $\times 300$.

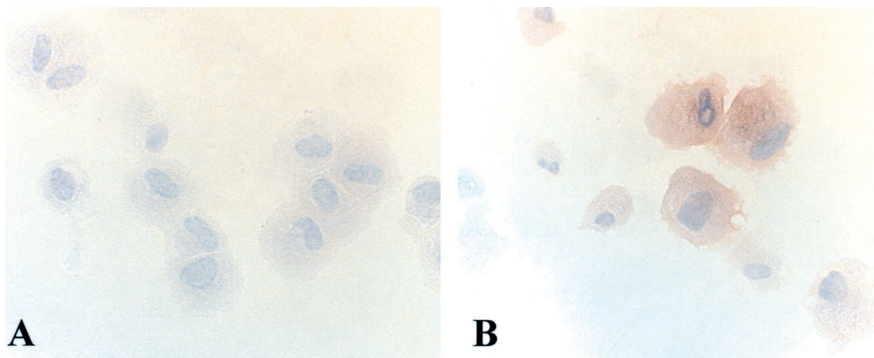


Fig. 2. Photomicrographs of cultured mesangial cells stained for hemopexin expression by monoclonal antihemopexin IgG antibody using peroxidase-conjugated rabbit antimouse and peroxidase-conjugated goat antirabbit IgG as a second and third step, respectively. It can be seen that, compared with unstimulated cells (A), predominant hemopexin staining occurs in mesangial cells after stimulation with tumor-necrosis factor- α (TNF- α) (B). Final magnification $\times 300$.

Netherlands) were used as control antigens. (These molecules were selected as they are known to contain hemopexin-like domains).

Detection of hemopexin mRNA in primary mesangium cells

RNA was isolated from frozen mesangium cells. Total RNA was isolated from either nonstimulated or TNF- α stimulated cells with or without prednisolone treatment, using Trizol (Life Technologies, Inc., Gaithersburg, MD, USA).

RNA isolation is followed by DNase treatment according to the protocol from the manufacturer (MessageClean Kit; Gen Hunter Corp., Nashville, TN, USA). The quality of isolated total RNA was checked using 1% agarose gel. DNase treatment was tested by PCR using a dinucleotide primer set D11S875 specific for genomic DNA (5'-ACTGTCCTCTCATCCTACTG-3' and

5'-TACAGAGCTGAGTTTGTAGC-3') and only those cases for which no PCR product was obtained were used for a further analysis.

The cDNA synthesis was primed with oligo(dT) using the protocol provided by the manufacturer (Gibco BRL, Eggenstein, Germany). Hemopexin primers were selected from the sequence presented in the GenBank (accession number M62642). Primer sequences used for the amplification were hemopexin (f 5'-GCACTCAGG TATATGTCTTCC-3'; r 5'-GGGCTCCTGACTTCAG GTCC-3') and the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (f 5'-CCATC ACTGCCACTCAGAAGACT-3', r 5'-TTACTCCTTG GAGGCCATGTAGG-3'). PCR for all primer sets was performed with 1 unit of Taq-polymerase (Pharmacia Biotech) and the reaction buffer provided by the manufacturer. The PCR program of hemopexin consisted of 30 cycles with a denaturation step of 30 seconds at 94°C,

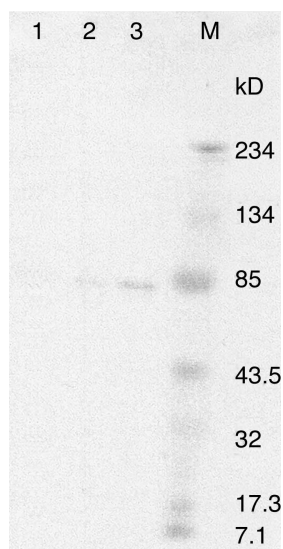


Fig. 3. Western blot stained for hemopexin showing 85 kD bands. It can be seen that an 85 kD band appears in supernatants of tumor necrosis factor- α (TNF- α)-stimulated cultures (lane 3). An identical band, although less prominent, can be seen in TNF- α -stimulated cultures after prednisolone treatment (lane 2), whereas no detectable 85 kD band can be seen in unstimulated cultures (lane 1). M is marker molecules.

an annealing step of 40 seconds at 57°C, and an extension step of 40 seconds at 72°C. The first denaturing step lasted 5 minutes and the final extension step lasted 7 minutes. Amplification of GAPDH was performed for 20 cycles and used as control to compare mRNA amounts.

RESULTS

The majority of cells stimulated with TNF- α stained positive for hemopexin using polyclonal antihemopexin IgG (Fig. 1B). In contrast nonstimulated cells, and to a lesser extent stimulated cells treated with prednisolone, stained predominantly negative (Fig. 1 A and C), although in several cells some membrane staining could be seen after prednisolone treatment (Fig. 1C). When the cells were stained for hemopexin using monoclonal antihemopexin IgG, again exclusively positive staining in TNF- α stimulated cells occurred (Fig. 2). Staining with antitransferrin IgG as a control antibody was negative (results not shown).

Inhibition of hemopexin synthesis by prednisolone is also reflected in the decreased release of this molecule in supernatants of stimulated cell culture treated with this drug. Supernatants of TNF- α stimulated cultures showed a clear 85 kD band in the Western blot, whereas a faint band was observed in supernatants from prednisolone-treated cultures after stimulation with TNF- α in vitro (Fig. 3). Western blots using antitransferrin antibody did not show a 85 kD band indicating that the bands shown

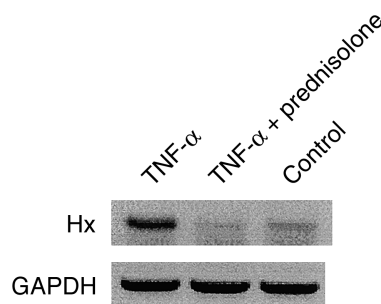


Fig. 4. Gel electrophoresis of hemopexin (Hx). Reverse transcription-polymerase chain reaction (RT-PCR) products from tumor necrosis factor- α (TNF- α)-stimulated mesangial cells with or without prednisolone (upper panel). The lower panel shows RT-PCR products from glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (housekeeping gene). Clear down-regulation of hemopexin expression following TNF- α stimulation with prednisolone versus TNF- α stimulation alone can be seen in the upper panel.

in Figure 3 are not due to nonspecific binding of IgG to hemopexin molecules (results not shown).

In addition, RT-PCR revealed a reduced signal for hemopexin in stimulated cells treated with prednisolone as compared with TNF- α -stimulated cells (Fig. 4).

The specificity of the monoclonal mouse anti hemopexin IgG was confirmed by Western blotting showing a single 80 kD band with purified plasma hemopexin, whereas control molecules (i.e., MMP-2 or MMP-9) stain negative (Fig. 5). Polyclonal antihemopexin IgG does not recognize MMP-2 or MMP-9 molecules in the Western blot (results not shown).

DISCUSSION

The aim of the present study was to confirm and extend our preliminary observation of a novel activity of mesangial cells in vitro (i.e., the production and release of hemopexin). It is clear from Figures 1 and 2 that TNF- α -stimulated cells synthesize hemopexin. In addition, it appeared that this synthesis is inhibited by prednisolone. Up-regulation of hemopexin by TNF- α and inhibition by corticosteroids in mesangial cell cultures is consistent with the mRNA signals (Fig. 4), supporting the idea that increased hemopexin protein expression relates to hemopexin transcription and synthesis in these cells. The finding that hemopexin is also detectable in culture supernatants of TNF- α -stimulated cells (and to a lesser extent in those of prednisolone-treated cells) (Fig. 3) is in line with the immunostaining data and points to release of hemopexin molecules by TNF- α -stimulated mesangium cells.

In view of decreased production of various molecules in other cell types due to corticosteroids [18–20], we feel that the prednisolone effect observed in this particular cell in vitro is not surprising; however, some discrepancy of the glucocorticoid action upon gene expression of acute

phase production exists. Thus, Baumann et al [21, 22] observed enhancement of acute phase proteins, including hemopexin in hepatocytes, after stimulation with conditioned monocyte medium, while dexamethasone was able to reduce the secretion of these proteins *in vitro*. On the other hand, interleukin-6 (IL-6)-induced hemopexin release in a rat hepatoma cell line was enhanced by transforming growth factor- β (TGF- β), whereas dexamethasone was up-regulating this TGF- β -dependent IL-6 response *in vitro* [23]. In addition, species differences were shown regarding the effect of glucocorticoid upon hemopexin production [24], whereas it has been shown that corticosteroids can enhance or diminish gene expression of acute phase reactants depending on their concentration [25]. *In vivo*, also, some discrepancy between glucocorticoid effects upon hemopexin production was observed. Thus, Eastman et al [25] observed abolishment of the hemopexin release after dexamethasone treatment of adrenalectomized rats, whereas Marinkovic et al [26] described IL-6-induced hemopexin induction in rats, which appeared to be enhanced by dexamethasone *in vivo*. Be this as it may, our previous (unpublished) experiments with human mesangium cells stimulated with IL-1 or IL-6 are in line with the present results showing prednisolone-dependent hemopexin release.

Although the molecular weight of hemopexin may differ in various hemopexin preparations due to different levels of glycosylation [1, 2], we feel that the data so far are consistent with hemopexin being the molecule involved. This does not mean, however, that stimulated mesangial cells produce necessarily an identical isoform of hemopexin as compared with plasma hemopexin. The fact that our antihemopexin monoclonal antibody does not recognize mesangial hemopexin in the Western blot (unpublished results), in contrast to purified plasma hemopexin (Fig. 5), may support this notion, although the possibility that this lack of recognition is due to the low amount of mesangial antigen used in the blotting assay can not be excluded. Extended concentration and purification studies with mesangial culture supernatants are necessary to settle this issue. Preliminary experiments, however, indicate that purified mesangial hemopexin isolated from the supernatants of stimulated mesangial cells shows serine protease activity, as is the case in plasma hemopexin [12].

These data taken together, we feel that stimulated mesangial cells like monocytes and fibroblasts [10] are potentially able to locally produce hemopexin. The relevance of this capability is unclear but may subserve a local protective function, through binding of free heme. Since accumulating evidence suggest that free heme can act as a proinflammatory signaling molecule, playing a role as catalyst in the oxidation of lipids, proteins, and DNA [27–30], local production of heme-binding molecules may protect the tissue from heme-mediated injury. Such a local protection has been recently suggested for neural

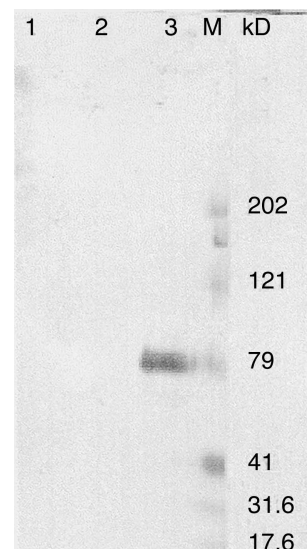


Fig. 5. Western blot stained for hemopexin (Hx). In a purified plasma hemopexin sample using monoclonal antihemopexin antibody, peroxidase-conjugated rabbit antimouse IgG, and peroxidase-conjugated goat antirabbit IgG as a second and third step, respectively, shows one band of approximately 80 kD band (lane 3). Control molecules [i.e., matrix metalloproteinase (MMP-2) (lane 1) or MMP-9 (lane 2)] stain negative. M is marker molecules.

tissue and peritoneal macrophages by Camborieu et al [10]. These authors show up-regulation of hemopexin production in peritoneal mononuclear cells following stimulation with proinflammatory agents such as lipopolysaccharide or IL-6, as well as in regenerating neural tissue. In addition it has been shown that hemopexin plays a key role in the homeostasis of nitric oxide radicals [31]. Since mesangium cells in particular are readily producing nitric oxide in the inflammatory condition [32, 33], nitric oxide radical scavenging by mesangial hemopexin may reflect an important local protective principle within the glomerular microvasculature.

Whatever the relevance of locally produced hemopexin may be in terms of heme scavenging, our previous observation that the protease-like activity of hemopexin can promote proteinuria [13] may be of interest for better understanding of the pathophysiology of corticosteroid-responsive proteinuria. The present findings open the possibility that in pathologic conditions this potentially proteinuria-inducing molecule can be produced in the glomerulus itself following local activation by cytokines *in vivo*.

In view of the suggested relationship between cellular immune responses and the pathogenesis of CRNS [34–36], it is tempting to speculate that a T cell-mediated event leading to relevance of proinflammatory cytokines may trigger mesangial hemopexin release promoting enhanced glomerular permeability. Further studies, using mesangial cells from biopsies of patients with CRNS in relapse

versus control subjects, may help to elucidate the possible relevance of mesangial hemopexin in this disorder.

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